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ORIGINAL PAPER

# *Pseudomonas* sp. strain AKM-P6 enhances tolerance of sorghum seedlings to elevated temperatures

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**Abstract** A thermotolerant strain AKM-P6 of *Pseudomonas* sp. possessing plant growth-promoting properties was isolated from rhizosphere of pigeon pea grown under semiarid conditions in India. The effect of inoculation with AKM-P6 on survival and growth of sorghum seedlings at elevated temperatures (ET) was investigated under sterile and nonsterile soil conditions. Inoculation with strain AKM-P6 helped sorghum (var CSV-15) seedlings to survive and to grow at elevated temperatures (47–50°C day/30–33°C night) up to 15 days while uninoculated plants died by the fifth day of exposure to elevated temperature. Under sterile and nonsterile conditions, significantly higher root and shoot biomass were recorded in inoculated seedlings as compared to uninoculated control at ET, but this difference was nonsignificant at ambient temperature. Inoculation induced the biosynthesis of high-molecular weight proteins in leaves under elevated temperature, reduced membrane injury, and improved the levels of cellular metabolites like proline, chlorophyll, sugars, amino acids, and proteins. Scanning electron microscopy studies confirmed the colonization and establishment of the organism on the root surface. The 16SrDNA sequence of the strain AMK-P6 showed 97% homology with that of *Pseudomonas aeruginosa* in the existing database. The results indicate that *Pseudomonas* sp. strain AKM-P6 can enhance tolerance of sorghum seedlings to elevated temperatures by inducing physiological and biochemical changes in the plant.

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## Introduction

The effects of global climate change, such as high temperatures, elevated levels of $CO_2$, and other greenhouse gasses on crop productivity are being extensively studied (Fischer et al. 2002; Drigo et al. 2008). Higher temperatures (higher than normal values observed in a region) influence photosynthetic rate, plant water relations, flowering, and fruit set in tropical and temperate crops (Abrol and Ingram 1996). In winter season, crops like wheat, an increase in mean minimum and maximum temperatures during reproductive stage adversely affected grain yields in Northern India (Sinha et al. 1998). Baker and Allen (1993) reported increased water requirements and decreased yields in rice, soybean, and citrus under higher maximum and minimum temperatures. For every 1°C rise in day/night temperature above 28/21°C, rice yield declined by 10%. Due to the unprecedented heat wave in Northern India during the year 2004, 4.4 million tons of loss in productivity was reported in wheat crop (Samra and Sing 2004). Breeding for suitable varieties, improved crop management, and changes in planting dates, etc., to some extent would help in overcoming the effects of climate change (Vanaja et al. 2007). However, evolving low cost methods, which can be easily adopted by small farmers, is a major challenge. Microorganisms are known to enhance the tolerance of plants to abiotic stresses like drought (Timmusk and Wagner 1999), chilling injury (Ait Barka et al. 2006), salinity (Han and Lee 2005), and metal toxicity (Dell' Amico et al. 2008). Redman et al. (2002) reported thermotolerance in *Dichanthelium lanuginosum* plants, by

symbiotic fungi *Curvularia* sp. When grown separately, neither the fungus nor the plants could tolerate the heat regime. The ability of the symbiotic fungus to confer heat tolerance to its host plant was related to a thermotolerant virus that infects fungus (Marquez et al. 2007). Srivastava et al. (2008) isolated a thermotolerant *Pseudomas putida* NBR10987 strain from drought stressed rhizosphere of chickpea. The thermotolerance of the strain was attributed to the over expression of stress sigma factor $\sigma^s$ and enhanced biofilm formation at high temperature. In the present investigation, an attempt was made to isolate thermotolerant strains of fluorescent *Pseudomonas* from semiarid regions and to study the ability of a selected thermotolerant fluorescent *Pseudomonas* sp. strain AKM-P6 to alleviate high temperature stress in sorghum seedlings. To the best of our knowledge, this is the first report on alleviation of high temperature stress in plants by *Pseudomonas*.

## Materials and methods

### Isolation and screening of thermotolerant fluorescent *Pseudomonas*

Fluorescent *Pseudomonas* sp. isolates were collected from rhizosphere soil samples of the plants growing under semiarid locations across India. The plants were uprooted along with bulk soil and brought immediately to the laboratory in polythene bags under refrigerated conditions for isolation of rhizobacteria within 48 h of uprooting. The bulk soil was removed by gently shaking the plants. The rhizosphere soil was collected by dipping the roots in sterile normal saline and kept on shaker for 30 min. The soil suspension was serially diluted and appropriate dilutions were spread plated on solid King's B medium. The plates were incubated at 28±2°C and fluorescent colonies were selected for further studies.

In order to screen the isolates for their thermotolerance, the overnight-grown broth cultures adjusted to optical density of 0.5 at 600 nm were inoculated into trypticase soy broth (TSB) and incubated at different temperatures (from 28°C to 50°C) on a rotary shaker at 120 rpm for 48 h. Optical density of the cultures incubated at different temperatures was recorded. The isolates showing growth (OD value >0.2 at 600 nm) at the highest temperature (50°C) were considered as thermotolerant for that particular temperature and selected for further studies.

Production of exopolysaccharides and induction of heat shock proteins in thermotolerant isolates.

The isolates able to tolerate high temperature (50°C) were monitored for their ability to produce EPS under ambient and elevated temperature. The extraction of exopolysaccharides (EPS) was done by centrifuging cultures raised in TSB at both ambient and elevated temperatures at 17,630×g for 25 min, and the supernatant was collected. The pellet was washed twice with 0.85% KCl for the complete recovery of EPS. The supernatants were pooled and checked for cell disruption by testing the presence of DNA by the method of Burton (1956). Protein content of the supernatant was determined (Lowery et al. 1951). The supernatant was filtered through 0.2 μm nitrocellulose membrane and dialyzed extensively against water at 4°C. The dialysate was centrifuged at 17,630×g to remove any insoluble material and mixed with 3 volumes of ice-cold absolute alcohol and kept overnight at 4°C. The precipitated EPS was sedimented by centrifugation at 8,815×g for 15 min, suspended in water, and further purified by repeating the dialysis and precipitation steps (Fett et al. 1989). Total carbohydrate contents of the precipitated EPS were determined according to Dubois et al. (1956).

In order to induce the heat shock, the cells were harvested from overnight-raised broth cultures by centrifugation at 2,655×g and washed twice with 50 mM sodium phosphate buffer (pH 7.0). The cells were resuspended in 25 ml of Luria Bertani broth ($10^7$CFU $ml^{-1}$) in Erlenmeyer flasks and subjected to heat shock (50°C) for 20 min in a shaking water bath. Immediately after heat shock, the flasks were cooled to room temperature and the cells were harvested by centrifugation at 2,655×g and washed with 50 mM sodium phosphate buffer. The pellet was suspended in sonication buffer and sonicated at 4°C for ten short bursts of 10 sec each with an interval of 30 sec. The cell lysate obtained by centrifugation at 8,815×g at 4°C was analyzed for heat shock proteins by sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE) (Sambrook et al. 1989). The protein profiles of isolates at ambient and elevated temperature were compared.

### Plant growth promoting properties at ambient and elevated temperature

The thermotolerant isolates were tested for plant growth-promoting properties like production of ammonia (Dey et al. 2004), siderophore (Schwyn and Neilands 1987), hydrogen cyanide (HCN; Bakker and Schipper 1987), indole acetic acid (IAA; Gordon and Weber 1951), and gibberellic acid (Holbrook et al. 1961) for P-solubilization (Mehta and Nautiyal 2001). All the tests were replicated six times at ambient as well as elevated temperature (50°C).

### Biochemical and molecular characterization of *Pseudomonas* strain AKM-P6

The isolate AKM-P6, selected on the basis of thermotolerance (EPS production) and plant growth promoting properties was monitored for Gram staining, capsule

formation, pigment production, oxidase and catalase activity, indole production, methyl red, Voges–Proskauer test, gelatin and starch hydrolysis, denitrifying activity and use of citrate, glycerol, sucrose, glucose, fructose, mannitol, lactose, xylose, galactose, mannose, rhamnose, sorbitol, maltose, and raffinose according to Holt et al. (1994).The antibiotic resistance profile of the isolate AKM-P6 was developed by testing resistance of the isolate to polymyxin, nalidixic acid, methicillin, cloxacillin, amoxycillin, gentamycin, ciprofloxacin, carbenicillin, chloramphenical, streptomycin, trimethoprim, vancomycin, tetracycline, erythromycin, and ampicillin; these assay were done on solid medium using antibiotic disks of different concentrations (Himedia, India) (Lalucat et al. 2006).

For molecular characterization, bacterial genomic DNA was isolated (Chen and Kuo 1993) and the 16SrDNA gene was amplified by PCR using universal forward (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse (5′AAG GAGGTGATCCAGCCGCA 3′) primers under standard conditions (initial denaturation 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 40 sec, extension at 72°C for 90 sec, and final extension at 72°C for 7 min). The PCR product (~1,500 bp) was purified and sequenced (Oscimum Bio Solutions, India). The sequence obtained was compared with the existing database of 16 SrDNA gene and submitted to GenBank.

## Plant growth studies

The protective effect of inoculated *Pseudomonas* strain AKM-P6 on sorghum seedlings exposed to elevated temperatures was studied under sterile and nonsterile soil conditions. Seeds of sorghum (var. CSV-15) were surface-sterilized with 0.1% $HgCl_2$ and 70% ethanol and coated with talc based formulation ($10^8$cells/g) of *Pseudomonas* strain AKM-P6 using 1% carboxymethyl cellulose as adhesive. The seeds were sown in 250 ml plastic cups (surface-sterilized) filled with 200 g sterile/nonsterile soil and maintained at 2/3 of the field capacity. Soil was collected from homogeneous horizon (0–20 cm) of Gunegal Research Farm, Central Research Institute for Dryland Agriculture, a semiarid region under rain-fed production system. The soil was air-dried and sieved (<2 mm) before being analyzed for the physicochemical properties. The soil contained 74% sand, 5% silt, 24% clay with 1.40 Mg $m^{-3}$ bulk density, 36.9% total porosity, and 37.9% water holding capacity; it had pH 7.0 and electrical conductivity of 0.103 ms. Organic C, total N, and total P content of soil were, 0.82 g/kg, 0.16 g/kg, and 0.07 g/kg, respectively The treatments included seed inoculation with and without strain AKM-P6 at elevated (47°C to 50°C day and 30°C to 33°C night) and ambient temperatures (34°C to 37°C day and 25°C to 28°C night). Each treatment included 5-days-old seedlings and was replicated six times. Seedlings received 6,000-lux illumination for 16 h. Shoot and root biomass were determined by harvesting fifteen-days-old seedlings (10 days after transfer to ET). Rhizosphere and endorhizosphere count, were determined by serially diluting 1 g of rhizosphere soil with successive plating on King's B medium amended with trimethoprim and vancomycin (35 μg/ml). The plates were incubated at 28±2°C for 48 h, and fluorescent colonies were counted. Bacterial colonization of root from inoculated and uninoculated seedlings exposed to elevated temperature was determined by scanning electron microscope. The root samples were fixed in 2.5% gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4°C and postfixed with 2% aqueous osmium tetroxide in the same buffer for 2 h. After the post fixation samples were dehydrated in series with graded alcohol and dried to critical point, which was checked by drying with electron microscopy science critical point dried unit. Then dried samples were mounted over the stubs with double-sided conductivity tape, and a thin layer of gold metal was applied over the sample using an automated sputter coater (JEOL JFC-1600) for about 3 min (Bozzola and Russell 1999). Finally, samples were analyzed by scanning electron microscope (Model: JOEL-JSM 5600, JAPAN) at various magnifications at Ruska Lab, SVUU, Hyderabad, India.

## Plant biochemical parameters

The experiment was repeated to study the mechanism of protection of seedlings exposed to ET by *Pseudomonas* strain AKM-P6. Eight days old (3 days after exposure to ET) seedlings were harvested and the contents of total sugars, chlorophyll, starch, proline, protein, and amino acid of seedlings determined along with high-molecular weight proteins.

The contents of sugars and amino acids were determined by incubating 1 g of leaf sample with methanol–chloroform–water (60:25:15 v/v) mixture at 60°C for 2 h. The samples were centrifuged at 8,815×g and the content of total sugars of the supernatant estimated by phenol sulfuric acid method (Dubois et al. 1956). The content of total amino acids was determined by heating 1 ml of the supernatant with 1 ml of 0.1 M acetate buffer and 1 ml of ninhydrin (5%) at 95°C for 5 min. The samples were cooled and absorbance was read at 570 nm (Chen et al. 2007). Free proline content was determined by the method of Bates et al. (1973). The leaf samples homogenized in 3% sulphosalicylic acid were centrifuged at 8,815×g and the supernatant was heated at 100°C after the addition of acidic ninhydrin. The samples were extracted with toluene, and the chromophore containing toluene was aspirated, cooled to room temperature, and absorbance was read at 520 nm. Starch analysis was determined by homogenizing leaf samples in 0.1 M phosphate buffer (pH 7.5) at 4°C and centrifuged at 8,815×g. The

pellet was suspended in dimethyl sulphoxide (DMSO): 8 M HCl (4:1 v/v) and agitated at 60°C for 30 min. After centrifugation at 8,815×g, the supernatant was treated with 100 µl of acidified iodine, incubated at room temp for 15 min, and the absorbance was read at 600 nm (Ait Barka et al. 2006). The determination of total chlorophyll was done by immersing leaf samples in DMSO and incubating them at 70°C for 4 h. The absorbance of the solution was then read at 645, 663, and 480 nm (Barnes et al. 1992). The detection of high-molecular weight proteins was carried out by grinding 1 g of leaf sample in 10 ml 100 mM potassium phosphate buffer containing 0.5 mM EDTA. The homogenate was centrifuged at 8,815×g for 15 min and the content of the supernatant determined by Bradford method (Bradford 1976). The proteins were resolved on 12% acrylamide gel by SDS-PAGE (McLellan et al. 2007). The experiment was replicated three times to see the variability among the replicates of same treatment.

The membrane injury index (MII) of leaf tissues was determined by recording electrolyte leakage in deionozed water at 50°C and 121°C (Ibrahim and Quick 2001). Leaf samples (0.1 g) were cut into disks of uniform size and submerged in 10 ml of deionozed water in test tubes and heated at 50°C for 30 min. The tubes were incubated overnight at room temperature, and the conductance was measured using a conductivity meter. The tubes were then autoclaved for 10 min at 121°C and the conductance was measured again.

### Statistical analysis

The data obtained was analyzed by analysis of variance and comparison among treatments was done by Tukey's multiple comparisons.

## Results

### Isolation and screening

A total of 46 pseudomonads were isolated on King's B medium (Table 1). Out of 46, five isolates (GAP-P4, AKM-P6, RMP-P6, PMP-P29, and JG-P46) could grow normally at high temperature (50°C). All the selected thermotolerant isolates produced EPS under ambient as well as at high temperature, and the production of EPS was much higher at high temperature (Table 2). Isolate AKM-P6 was the best producer of EPS under normal as well as stressed conditions. Induction of high-molecular weight 60 KDa to 70 KDa proteins was observed in all the five thermotolerant isolates under elevated temperature.

Plant growth promoting (PGP) properties of five thermotolerant strains were studied under ambient and elevated temperature. All isolates produced IAA, gibberellins, and ammonia under ambient as well as elevated temperature. However, significant reduction in PGP traits was observed under elevated temperature (Table 2). Isolate AKM-P6 produced maximum amount of IAA (56.91± 1.077 µg/mg protein) under ambient temperature, closely followed by isolate RMP-P6 (56.22±0.593) and JG-P46 (55.29±0.808). Under elevated temperature, isolate JG-P46 was the best producer of IAA (29.77±1.567 µg/mg protein) followed by RMP-P6 (25.90±2.96) and AKM-P6 (25.8± 2.914). Isolate AKM-P6 was the best producer of gibberellic acid under ambient (0.659±0.006 µg/mg protein) and elevated temperature (0.189±0.001 µg/mg protein). Amount of P-solubilized was also the maximum in isolate AKM-P6 both under ambient (60.33±2.96 µg/ml) and elevated (40.0±0.577 µg/ml) temperature, whereas isolate GAP-P4 could not solubilize P at all. Further, concentration of produced gibberellic acid and amount of P-solubilized by thermotolerant AKM-P6 under elevated temperature was significantly higher than values of all other isolates under ambient temperature. Hydrogen cyanide production was observed in two isolates (AKM-P6 and RMP-P6) and siderophore production was only observed in AKM-P6 under ambient and elevated temperature. Ammonia production was observed in all isolates under both ambient and elevated temperature. Isolate AKM-P6 was selected for plant growth studies since it showed the best plant growth-promoting traits (Table 2). Microscopic studies revealed strain AKM-P6 as Gram-negative, motile with capsulated rods. The isolate showed presence of oxidase and catalase activity, exhibited denitrification, gelatin degradation, and arginin hydrolysis and could utilize citrate, glycerol, ribose, fructose, glucose, mannitol, and lactose (Table 3). The

**Table 1** Sites in India of soil samplings for the isolation of stress tolerant *Pseudomonas* sp. strains

| S.N0 | Crop | Location | State | Soil type | Climate | Total number of *Pseudomonas* sp. | Thermotolerant strains |
|---|---|---|---|---|---|---|---|
| 1 | Chick pea | Gunegal | Andhra Pradesh | Alfisol | Semiarid | 5 | GAP-P4 |
| 2 | Pigeon pea | Akola | Maharastra | Vertisol | Semiarid | 16 | AKM-P6 |
| 3 | Soya bean | Rewa | Madhya Pradesh | Vertisol | Subhumid | 12 | RMP-P6 |
| 4 | Sorghum | Junagadh | Gujarat | Vertisol | Semiarid | 5 | PMP-P29 |
| 5 | Sorghum | Parbhani | Madhya Pradesh | Vertisol | Semiarid | 8 | JG-P46 |

**Table 2** Plant growth-promoting traits and exopolysaccharide (EPS) production by thermotolerant isolates at ambient and elevated temperature

| Strains | Temperature | IAA μgmg$^{-1}$ protein | Gibberellins μg mg$^{-1}$ Protein | P-solubilization μg ml$^{-1}$ | HCN | Siderophore | Ammonia | EPS mgmg$^{-1}$ Protein |
|---|---|---|---|---|---|---|---|---|
| GAP-P4 | AT | 11.00±0.99 | 0.218±0.002 | ND | − | − | ++ | 17.50±0.404 |
| | ET | 04.66±0.584 | 0.008±0.004 | ND | − | − | + | 42.83±1.85 |
| AKM-P6 | AT | 56.91±1.077 | 0.659±0.006 | 60.33±2.96 | ++++ | +++ | ++ | 27.50±1.19 |
| | ET | 25.80±2.914 | 0.189±0.001 | 40.00±0.577 | ++ | + | + | 51.24±1.92 |
| RMP-P6 | AT | 56.22±0.593 | 0.317±0.002 | 39.33±0.088 | ++ | − | ++ | 23.73±1.53 |
| | ET | 25.90±2.96 | 0.114±0.004 | 26.33±2.18 | + | − | + | 39.57±0.983 |
| PMP-P29 | AT | 10.92±0.707 | 0.224±0.002 | 41.67±3.28 | − | − | ++ | 12.93±0.284 |
| | ET | 4.06±0.088 | 0.089±0.0008 | 28.08±3.12 | − | − | + | 22.57±3.62 |
| JG-P46 | AT | 55.29±0.808 | 0.085±0.006 | 31.00±1.15 | − | − | ++ | 16.60±0.264 |
| | ET | 29.77±1.567 | 0.029±0.001 | 16.00±2.08 | − | − | + | 32.57±0.606 |

*AT* ambient temperature, *ET* elevated temperature, + presence of trait, − absence of trait, +fair, ++ good, +++ very good, ++++ excellent. Values are the means of six replicates with ± SEM

**Table 3** Phenotypic characterization of *Pseudomonas* strains

| Test | GAP-P4 | AKM-P6 | RMP-P6 | PMP-29 | JG-P46 |
|---|---|---|---|---|---|
| Colony morphology | | | | | |
| (a) Smooth | − | + | + | + | + |
| (b)Wrinkled | + | − | − | − | − |
| (c)Round | + | + | + | + | + |
| (d)Convex | + | + | + | + | + |
| Biochemical tests | | | | | |
| Indole | − | − | − | − | − |
| Methyl red | − | − | − | − | − |
| Voges–Proskauer | − | − | − | − | − |
| Citrate | + | + | + | + | + |
| Oxidase | + | + | + | + | + |
| Catalase | + | + | + | + | + |
| Starch | + | − | − | − | − |
| Denitrification | + | + | − | − | − |
| Sugar fermentation | | | | | |
| Glycerol | + | + | + | + | + |
| D-Galactose | − | − | − | + | − |
| D-Glucose | + | + | + | + | + |
| D-Fructose | + | + | + | + | + |
| D-Mannose | − | − | − | + | − |
| Rhamnose | − | − | − | − | − |
| Mannitol | + | + | − | + | − |
| Sorbitol | − | − | − | + | − |
| Maltose | + | − | + | − | + |
| Lactose | + | + | + | − | − |
| Sucrose | − | − | − | + | − |
| D-Raffinose | − | − | − | + | − |
| Arginin | − | + | − | − | − |

**Table 4** Blast similarity index of 16S rDNA gene of *Pseudomonas* sp. strain AMK-P6

| Accession number | Maximum score | Total score | Query coverage | E-value | Maximum identity |
|---|---|---|---|---|---|
| AY486356.1 | 1945 | 1945 | 99% | 0.0 | 97% |
| FJ652616.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| FJ652615.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| FJ652614.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| GQ180118.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| GQ180117.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| GQ037324.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| FJ948174.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| FJ870126.1 | 1943 | 1943 | 99% | 0.0 | 97% |
| FJ665510.1 | 1943 | 1943 | 99% | 0.0 | 97% |

strain *Pseudomonas* AKM-P6 showed resistance to nalidixic acid, cloxacillin, methicillin, amoxycillin, chloramphenical, trimethoprim, vancomycin, tetracycline, erythromycin, and ampicillin. Molecular characterization of the strain was done on the basis of 16SrDNA gene sequence that showed 97% homology with that of *P. aeruginosa* in the existing database (Table 4). The sequence was submitted to GenBank under the accession no FJ654697.

## Plant growth studies under sterile and unsterile conditions

The effect of sorghum seedlings inoculation with selected *Pseudomonas* sp. strain AKM-P6 was studied under sterile as well as nonsterile conditions under ambient and elevated temperature. Inoculation improved shoot, root length, and dry biomass of plant under elevated temperature conditions with respect to values of the uninoculated plants which showed lower values when the temperatures was increased (Figs. 1 and 2). The uninoculated plants started wilting after 2 days of exposure to elevated temperature and died completely at the end of fifth day, both under sterile and nonsterile conditions (Figs. 1, 2 and 3). However, inoculated plants survived up to 15 days (sterile conditions) to 18 days (nonsterile conditions) after exposure to elevated temperature and started dying thereafter. Rhizosphere colonization by the inoculated strain was studied by plating appropriate serial dilutions of rhizosphere soil and mascerated root sample on King's B medium containing antibiotics (trimethoprim and vancomycin). The inoculated strain colonized the sorghum rhizosphere after 15 days of inoculation as indicated by count of the fluorescent pseudomonads, both in the rhizosphere and endorhizosphere, and also by scanning electron microscopy of root samples (Fig. 4). Under sterile conditions, population of fluorescent pseudomonads in rhizosphere and endorhizosphere of inoculated plant was $4\times10^7$ cfu/g soil and $2\times10^3$ cfu/g root under ambient temperature and $2\times10^4$ cfu/g soil and $2\times10^2$ cfu/g root under elevated temperature, respectively. Under nonsterile conditions, counts of fluorescent pseudomonads in rhizosphere and endorhizosphere of inoculated plants were $1.6\times10^7$ cfu/g soil and $2.3\times10^3$ cfu/g

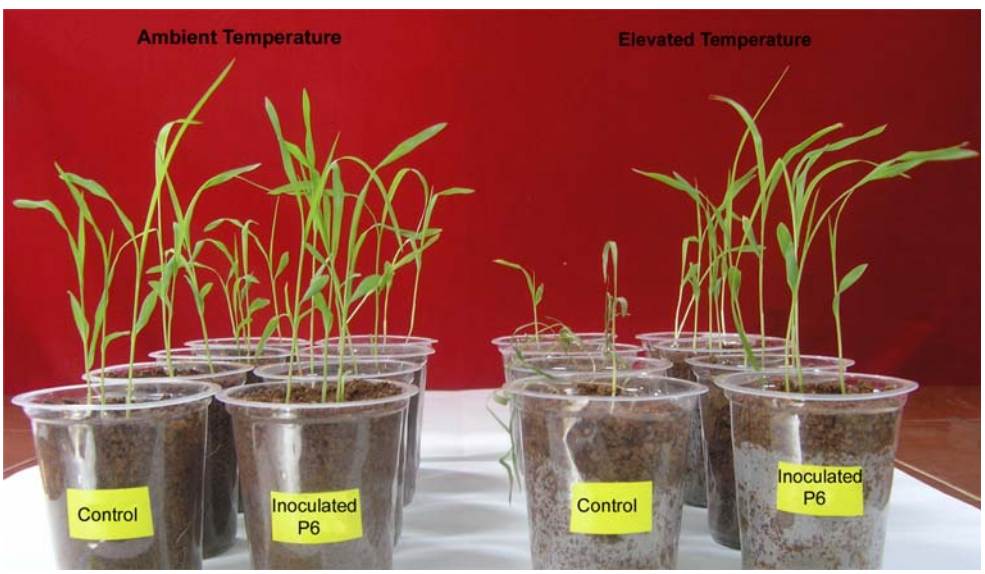


**Fig. 1** Effect of *Pseudomonas* sp. strain AKM-P6 inoculation on fifteen-days-old sorghum seedlings at ambient and elevated temperatures

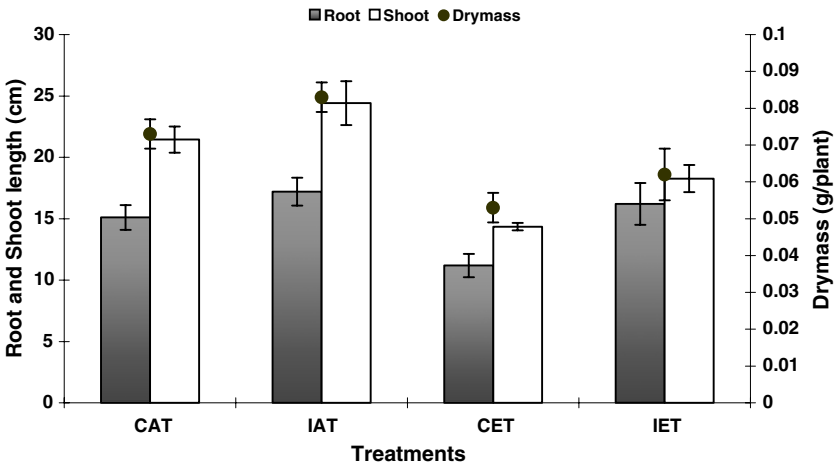


**Fig. 2** Shoot, root length, and biomass of sorghum seedlings inoculated with *Pseudomonas* sp. strain AKM-P6 exposed to ambient and elevated temperature under sterile soil conditions. (*CET* control at elevated temperature, *IET* inoculated at elevated temperature, *CAT* control at ambient temperature, *IAT* inoculated at ambient temperature)

root under ambient temperature and $2.5\times10^4$ cfu/g soil and $1.1\times10^2$ cfu/g root under elevated temperature, respectively.

## Biochemical parameters

The effect of inoculation on biochemical status of plant under ambient and elevated temperature was studied under sterile and nonsterile conditions. Under sterile conditions, inoculation significantly enhanced the contents of chlorophyll, total sugars, starch, amino acids, and protein in the shoots of sorghum seedlings under ambient temperature (Table 5). Exposure of inoculated and uninoculated seedlings to elevated temperature significantly decreased chlorophyll, total sugars, and protein contents but not the amino acid contents of inoculated seedlings at elevated temperature. A significant decrease in the starch content of uninoculated seedlings was observed on exposure to elevated temperature, whereas the starch content of inoculated seedlings significantly increased on exposure to elevated temperature. Membrane injury index as determined by electrolyte leakage showed a drastic increase on exposure of seedlings (inoculated and uninoculated) to elevated temperature; however, bacterial inoculation reduced the MII significantly over control treatment under elevated temperature (Table 6). Inoculation improved proline concentration of sorghum seedlings under ambient temperature and elevated temperature with the latter increase being higher than the former increase. All biochemical parameters of inoculated seedlings exposed to elevated temperature were significantly improved as compared to uninoculated seedlings exposed to elevated temperature and uninoculated seedlings at ambient temperature.

Under nonsterile conditions, similar trend, as observed under sterile conditions were observed (with a few exceptions; Table 4). Under nonsterile conditions, chlorophyll contents of inoculated seedlings under elevated temperature were significantly higher than those of uninoculated seedlings at ambient temperature. Amino acid content of uninoculated seedlings decreased significantly on exposure to elevated temperature under nonsterile

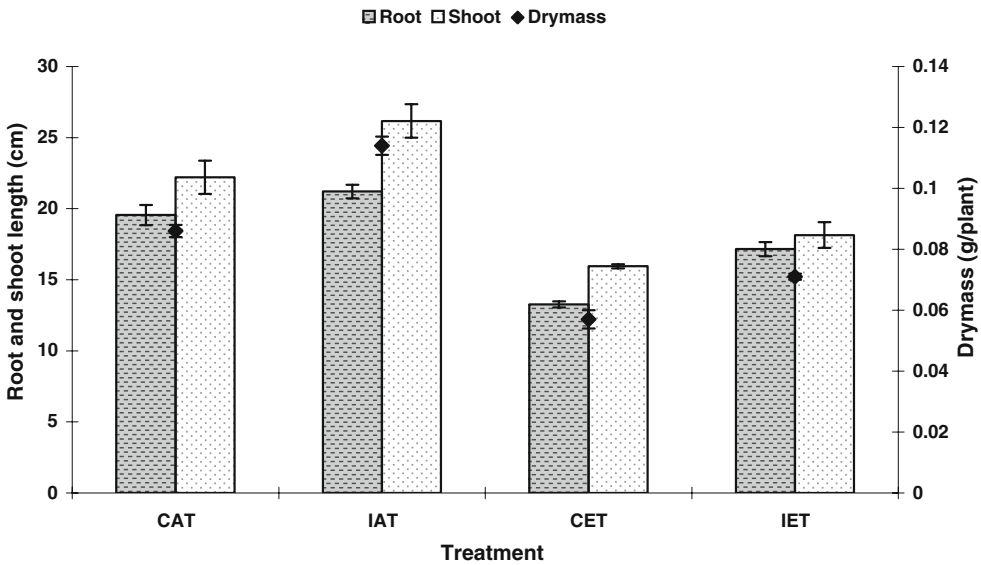


**Fig. 3** Shoot, root length, and biomass of sorghum seedlings inoculated with *Pseudomonas* sp. strain AKM-P6 exposed to ambient and elevated temperature under nonsterile soil conditions. (*CET* control at elevated temperature, *IET* inoculated at elevated temperature, *CAT* control at ambient temperature, *IAT* inoculated at ambient temperature)

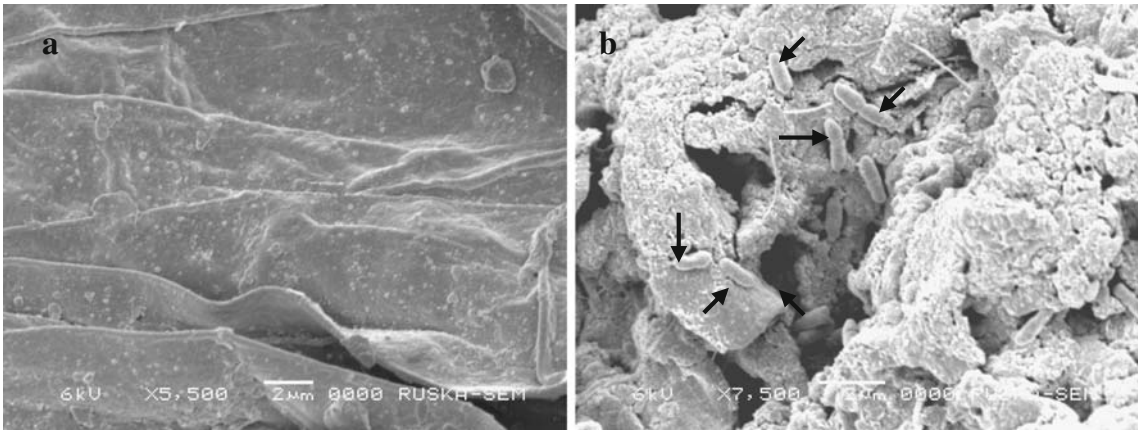


**Fig. 4** Scanning electron microscopy of sorghum seedlings showing the presence of bacterial cells on the root (**a** uninoculated, **b** inoculated); *arrows* indicate presence of bacteria

conditions whereas there was no statistically significant difference between amino acid concentrations of inoculated seedlings at elevated temperature and uninoculated seedlings at ambient conditions under nonsterile conditions.

### Induction of heat shock proteins

Induction of heat shock proteins (HSPs) in sorghum seedlings under sterile conditions was studied by SDS-PAGE. Protein profiles of inoculated and uninoculated sorghum seedlings exposed to ambient and elevated temperature revealed the presence of three additional polypeptides (~70.4 ~60.8 and ~55.1 KDa) in the seedlings exposed to elevated temperature (Fig. 5). The two additional polypeptides (~70.4 and ~60.8 KDa) bands observed in uninoculated seedlings on exposure to elevated temperature had a lower intensity than that of respective bands of inoculated seedlings at elevated temperature. The intensity of bands of many polypeptides was markedly reduced on exposure of seedlings to elevated temperature. Further, no variability was observed in the banding patterns on replicating the experiment.

## Discussion

The adverse effect in plants by abiotic stresses like drought, chilling, salinity, and metal toxicity is alleviated by microbial inoculation (Timmusk and Wagner 1999; Redman et al. 2002; Han and Lee 2005; Ait Barka et al. 2006; Marquez et al. 2007; Dell' Amico et al. 2008); here, we present the first evidence for the mitigation of stress by high temperature in sorghum by a bacterium.

Tolerance of plants to abiotic stresses depends on the type of plant and environment factors. A number of rhizobacteria including *Pseudomonas* (Hong et al. 1991) are known to promote plant growth. Timmusk and Wagner (1999) demonstrated that inoculation with *Paenibacillus polymyxa* protected *Arabidopsis thaliana* from drought stress by increasing the expression of stress-induced gene *Erd15*. We have isolated the used strains from the rhizosphere of millet plants grown under semiarid conditions, characterized by high temperature values; it is possible that the isolated microorganisms have evolved mechanisms to survive to the stressed environment. Indeed,

**Table 5** Effect of inoculation of *Pseudomonas* sp. strain AKM-P6 on biochemical and physiological parameters of sorghum seedlings at ambient and elevated temperatures under sterile soil conditions

| Treatment | MII (%) | Chlorophyll (mg $gFW^{-1}$) | Total sugars (mg $gDW^{-1}$) | Proline (µmol $gFW^{-1}$) | Starch (mg $gFW^{-1}$) | Amino acid (µmol $gDW^{-1}$) | Total protein (mg $gDW^{-1}$) |
|---|---|---|---|---|---|---|---|
| CAT | $48.18^{a}$ | $7.76^{a}$ | $88.55^{a}$ | $2.19^{a}$ | $0.023^{a}$ | $36.22^{a}$ | $74.97^{a}$ |
| IAT | $46.54^{a}$ | $11.59^{b}$ | $105.33^{b}$ | $2.77^{a}$ | $0.069^{b}$ | $38.77^{b}$ | $84.91^{b}$ |
| CET | $74.14^{b}$ | $6.18^{c}$ | $71.47^{c}$ | $8.02^{b}$ | $0.020^{c}$ | $28.96^{c}$ | $53.94^{c}$ |
| IET | $63.18^{c}$ | $8.62^{da}$ | $86.06^{da}$ | $9.59^{c}$ | $0.075^{d}$ | $35.20^{dab}$ | $62.48^{d}$ |

*CAT* control ambient temperature, *CET* control-elevated temperature, *IAT* inoculated ambient temperature, *IET* inoculated elevated temperature, *MII* membrane injury index

Values with different letters are significantly different at $P < 0.05$

**Table 6** Effect of inoculation of *Pseudomonas* sp. strain AKM-P6 on biochemical and physiological parameters of sorghum seedlings at ambient and elevated temperatures under nonsterile soil conditions

| Treatment | MII (%) | Chlorophyll (mg $gFW^{-1}$) | Total sugars (mg $gDW^{-1}$) | Proline (μmol $gFW^{-1}$) | Starch (mg $gFW^{-1}$) | Amino acid (μmol $gDW^{-1}$) | Total protein (mg $gDW^{-1}$) |
|---|---|---|---|---|---|---|---|
| CAT | $48.08^{a}$ | $8.12^{a}$ | $91.52^{a}$ | $3.83^{a}$ | $0.053^{a}$ | $41.41^{a}$ | $83.39^{a}$ |
| IAT | $45.27^{b}$ | $12.42^{b}$ | $122.80^{b}$ | $3.98^{a}$ | $0.084^{b}$ | $47.10^{b}$ | $94.08^{b}$ |
| CET | $72.33^{c}$ | $7.14^{c}$ | $78.33^{c}$ | $8.66^{b}$ | $0.045^{ca}$ | $39.13^{ca}$ | $60.90^{c}$ |
| IET | $62.4^{d}$ | $9.11^{d}$ | $92.23^{da}$ | $10.68^{c}$ | $0.098^{d}$ | $43.49^{dac}$ | $72.92^{d}$ |

*CAT* control ambient temperature, *CET* control-elevated temperature, *IAT* inoculated ambient temperature, *IET* inoculated elevated temperature, *MII* membrane injury index

Values with different letters are significantly different at $P < 0.05$

of the 46 isolates, five could resist high temperature (50°C) probably due to production of EPS and accumulation of HSPs, with the former probably forming a microsheath around microbial cells, thus protecting them from surrounding harsh conditions. Hartel and Alexandre (1986) observed a significant correlation between the amount of EPS produced by cowpea *Bradyrhizobium* strains and their desiccation tolerance. Heat shock proteins are membrane-stabilizing proteins induced under stress conditions and confer thermotolerance to elevated temperature (Lindquist 1986; Gouesbet et al. 2002). The used thermotolerant *Pseudomonas* sp. strain AKM-P6 produced EPS under temperature stress and also showed plant growth promoting traits like production of phytohormones, HCN, ammonia, siderophore, and P-solubilization. Dell' Amico et al. (2008) showed that inoculation with cadmium resistant strains *Pseudomonas tolaasi* and *Pseudomonas fluorescens* enabled *Brassica napus* to grow under cadmium stress because of the production of indole acetic acid, siderophores, and ACC deaminase, which protected the plants against cadmium stress. Under sterile and nonsterile conditions, thermotolerant strain AKM-P6 efficiently colonized the rhizosphere of the sorghum plants as indicated by rhizosphere and endorhizosphere counts and scanning electron microscopy. Successful colonization of plant roots by the introduced strain suggested a close bacterial–plant association that may be beneficial to plants. Inoculation with AKM-P6 influenced biochemical and physiological parameters of seedlings, both at ambient and elevated temperature, as indicated by increased levels of chlorophyll, total sugars, starch, proline, protein, and amino acid contents in the inoculated seedlings. Lower electrolyte leakage in inoculated plants suggested protection of membrane integrity by bacterium, probably due to alterations of plant lipid metabolism. Proline content of uninoculated plants increased by more than threefolds on exposure to elevated temperature indicating a role of this amino acid in stressed sorghum seedlings as it has been observed in grapevine inoculated with *Burkholderia phytofirmans* strain PsJN and subjected to chilling stress (Ait Barka et al. 2006). Bano and Fatima (2009) observed that the salt tolerance in *Zea mays* inoculated with *Rhizobium* and *Pseudomonas* was mediated by the decrease in electrolyte leakage and the increase in proline production

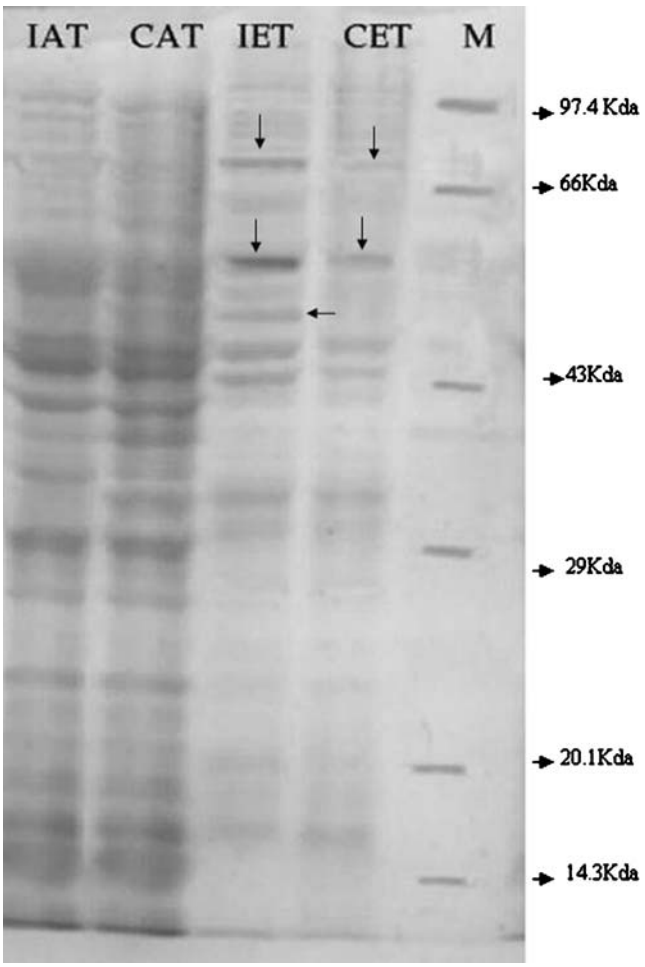


**Fig. 5** Induction of high-molecular weight proteins in leaves of sorghum seedlings inoculated with *Pseudomonas* sp. strain AKM-P6 at elevated temperatures (*CET* control at elevated temperature, *CAT* control at ambient temperature, *IET* inoculated at elevated temperature, *IAT* inoculated at ambient temperature, *M* marker); *arrows* indicates ~70.4 ~60.8 (IET and CET) and ~55.1 KDa (IET)

and maintenance of water content of leaves with selective uptake of K ions. In our study inoculation did not affect plant growth and biomass at ambient temperatures, whereas at elevated temperatures, significant difference in plant growth and survival were observed due to inoculation, probably because *Pseudomonas* inoculation triggers some stress responsive mechanisms that enable the plants to tolerate high temperatures. The SDS-PAGE analysis of leaf proteins revealed the presence of three high-molecular weight polypeptides in the inoculated plants exposed to elevated temperatures. Induction of heat shock proteins has been reported in several plants (Howarth 1991). McLellan et al. (2007) observed that the rhizosphere fungus *Paraphaeosphaeria quadriseptata* enhanced thermotolerance of *Arabidopsis thaliana* through induction of HSP101 and HSP70 proteins. Therefore, we suggest that the inoculation with *Pseudomonas* sp. strain AKM-P6 enhanced the tolerance of sorghum seedlings to high temperature due to the synthesis of high-molecular weight proteins and also to the improvement of cellular metabolites levels. This finding suggests the possible role of microorganisms in mitigating adverse effects of climate changes on crop growth and may lead to development of microbial inocula to mitigate such effects. However, further studies are required under greenhouse and field conditions and the mechanism of protection have to be elucidated.

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